PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 9/02, 1/20, 15/00, 15/09, 15/63, 15/82, C07H 21/04

A1

(11) International Publication Number: WO 98/04685

(43) International Publication Date: 5 February 1998 (05.02.98)

(21) International Application Number: PCT/US97/14351

(22) International Filing Date: 25 July 1997 (25.07.97)

(30) Priority Data:
60/022,604 25 July 1996 (25.07.96) US

(71) Applicant (*for all designated States except US*): AMERICAN CYANAMID COMPANY [US/US]; 1 Cyanamid Plaza, Wayne, NJ 07470 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): STURNER, Stephen [US/US]; 250 High Street, Newtown, PA 18940 (US). HIRAYAMA, Lynne, Miyo [US/US]; 221 Natrena Avenue, Mercerville, NJ 08619 (US). SINGH, Bijay [-/US]; 12 Abemarle Road, Hamilton Square, NJ 08690 (US). BASCOMB, Newell [US/US]; 9 Gallo Court, Lawrenceville, NJ 08648 (US).

(74) Agents: ROBINSON, Joseph, R. et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HPPD GENE AND INHIBITORS

(57) Abstract

The nucleic acid sequence encoding 4-hydroxyphenylpyruvate dioxygenase (HPPD) from Arabidopsis thaliana is disclosed. Also, a vector containing the DNA coding for HPPD, and transformed cells are disclosed. In addition, the description teaches of methods for the identification herbicide resistant HPPD, and herbicides which are inhibitors of HPPD as well as a method of conferring herbicide resistant on plants. Furthermore, the description teaches of a method for weed control.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

HPPD GENE AND INHIBITORS

Field of the Invention

This invention pertains to DNA encoding 4-hydroxyphenylpyruvate dioxygenase (HPPD), HPPD-inhibiting herbicides, and methods for screening compounds to identify HPPD-inhibiting herbicides. The invention also pertains to HPPD variants that are resistant to the inhibitory action of herbicides, methods for screening for HPPD variants, and plants comprising herbicide-resistant HPPD.

Background of the Invention

In plants, 4-hydroxypenylpyruvate dioxygenase (HPPD, EC 1.13.11.27) is a key enzyme in the biosynthesis of plastoquinones and tocopherols. 4-hydroxyphenylpyruvate acid (derived from chorismic acid via the shikimate pathway) is oxidized and decarboxylated by HPPD to yield homogentisic acid (Fiedler and Schultz, *Dev. Plant Biol.* 8:537, 1982; Fiedler et al., *Planta* 155:511, 1982). Subsequent polyprenylation and decarboxylation of homogentisic acid results in an array of plastoquinones and tocopherols.

In animals, HPPD is involved in tyrosine catabolism. A genetic deficiency in this pathway in humans and mice leads to hereditary tyrosinemia type 1. This disease can be treated by NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione, an inhibitor of HPPD, which prevents the buildup of intermediates of tyrosine catabolism that are hepatotoxic (Ellis et al., *Tox. and Appl. Pharm.* 133:12, 1995).

Since plastoquinones and tocopherols are essential compounds for plants, inhibitors of this enzyme are potential herbicides. One class of HPPD inhibitors, the triketones, have recently been shown to possess herbicidal activity (Prisbylia et al., *Brighton Crop Protection Conference: Weeds*, British Crop Protection Council, Surrey, UK, pp 731-738, 1993; Schulz et al., *FEBS Letts.* 318:162, 1993). The corn-selective herbicidesulcotrione(2-(2-chloro-4-methanesulfonylbenzoyl)-1,3-cyclohexanedione)causes

strong bleaching in susceptible plants accompanied by a loss of carotenoids and chlorophyll with an increase in phytoene and tyrosine (Barta et al., *Pest.Sci.* 45:286, 1995; Soeda et al., *Pestic.Biochem.Physiol.* 29:35, 1987; Mayonado et al., *Pestic.Biochem.Physiol.* 35:138, 1989). Treatment of *Lemna* with sulcotrione severely inhibited growth and the herbicidal effect could be abolished with homogentisic acid. The partially purified enzyme extracted from maize was shown to be severely inhibited by sulcotrione with a calculated IC_{50} of 45 nM (Schulz et al., 1993, *supra*). Analysis of partially purified HPPD from barnyardgrass *(Echinochloa crus-galli L.)* showed sulcotrione to be a potent competitive inhibitor of the enzyme with a K_i of 9.8 nM (Secor, *Plant Physiol.* 106:1429, 1994). Canadian Patent Application No. 2,116,421 describes the identification of HPPD inhibitors derived from 2-benzoylcyclohexamine 1,3-diones.

An albino mutant (*psdl*) isolated from a T-DNA tagged *Arabidopsis* population was originally selected by virtue of a severe pigment deficiency, which was thought to be due to a defect in carotenoid biosynthetic genes (Norris et al., *Plant Cell* 7:2139, 1995). When the albino *psdl* mutant was germinated on MS2 medium and subsequently transferred to MS2 medium supplemented with either 4-hydroxyphenylpyruvate (OHPP) or homogentisic acid (HGA), the plants greened on HGA but not OHPP. Further analysis of this mutant indicated that the defect causing the albino phenotype is not due to a mutation in a carotenoid biosynthesis enzyme directly, but rather results from a mutation in HPPD that prevents the biosynthesis of a plastoquinone essential for carotenoid biosynthesis.

Despite the importance of this pathway in plants, genes encoding the plant enzymes for plastoquinone and tocopherol biosynthesis have not previously been isolated. Thus, there is a need in the art for methods and compositions that provide HPPD genes, HPPD inhibitors useful as herbicides, and herbicide-resistant HPPD variants. The present inventors have isolated the gene encoding plant HPPD, have expressed it in *E. coli*, and have demonstrated that bacterially expressed plant HPPD is enzymatically active and that its enzymatic activity is inhibited by triketone herbicides.

Brief Description of the Drawings

Figure 1 is an illustration of the amino acid sequence of 4-hydroxyphenylpyruvate dioxygenase (HPPD) from *Arabidopsis thaliana* (AtHPPD) and

shows the alignment of this sequence with related sequences from mouse, human, pig, and *Streptomyces avermitilis* (S.Aver).

Figure 2 is a graphic illustration of the production of brown pigment by *E. coli* transformed with the *Arabidopsis* HPPD gene ("Arabidopsis") compared with *E. coli* transformed with a control vector ("plasmid"). The effect on pigment formation of adding increasing concentrations of tyrosine to the culture medium is shown.

Figure 3A is an illustration of an HPLC elution profile of medium from *E. coli* transformed with a control vector. Figure 3B is an illustration of an HPLC elution profile of medium from *E. coli* transformed with the *Arabidopsis* HPPD gene. The elution position of authentic homogentisic acid standard is indicated by an arrow. The insert in Figure 3B is an illustration of the absorption spectrum of the homogentisic acid peak.

Figure 4 is a graphic illustration of the effect of increasing concentrations of sulcotrione on the HPPD enzymatic activity of cell extracts derived from *E. coli* transformed with the *Arabidopsis* HPPD gene.

Summary of the Invention

The present invention provides purified isolated nucleic acids encoding plant 4-hydroxyphenylpyruvate dioxygenase (HPPD), in particular HPPD derived from *Arabidopsis thaliana*, as well as sequence-conservative variants and function-conservative variants thereof; DNA vectors comprising HPPD-encoding nucleic acid operably linked to a transcription regulatory element; and cells comprising the HPPD vectors, including without limitation bacterial, fungal, plant, insect, and mammalian cells. In one embodiment, a bacterial cell expressing high levels of plant HPPD is provided. Also encompassed are HPPD polypeptides and enzymatically active fragments derived therefrom.

In another aspect, the invention provides methods for identifying herbicides/HPPD inhibitors, which are carried out by:

(a) providing a microbial cell expressing plant HPPD;

(b) incubating the cell in the presence of a test compound to form a test culture, and in the absence of a test compound to form a control culture;

(c) monitoring the level of homogentisic acid, or oxidation products thereof, in the test and control cultures; and

(d) identifying as a compound that inhibits HPPD any compound that reduces the level of homogentisic acid, or oxidation products thereof, in the test culture relative to the control culture. In the above methods, the monitoring step may be achieved, for example, by measuring the absorbance of said cultures at 450 nm or by visually detecting formation of a brown pigment. Alternatively, an inhibitor is identified as a compound that inhibits the growth of the test culture, wherein the inhibition can be reversed by the addition of homogentistic acid to the culture.

In a further aspect, the invention provides methods for identifying herbicide-resistant HPPD variants, which are carried out by

(a) providing a population of cells expressing plant HPPD;

(b) mutagenizing the population of cells;

(c) contacting the mutagenized population of cells with an herbicide, under conditions inhibitory for the growth of non-mutagenized cells;

(d) recovering cells resistant to the inhibitory effects of the herbicide on growth and/or pigment formation; and

(e) sequencing HPPD-encoding nucleic acid from the recovered cells.

Alternatively, DNA encoding HPPD is subjected to random or site-directed mutagenesis *in vitro*, followed by expression in a heterologous cell and screening or selection of cells that exhibit herbicide resistance.

In yet another aspect, the invention encompasses variant HPPD proteins that are herbicide-resistant. Preferably, an herbicide-resistant HPPD variant protein, when expressed, in a cell that requires HPPD activity for viability, exhibits

(i) catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or catalytic activity in combination with any herbicide resistant HPPD variant protein also expressed in the cell, which may be the same as or different than the first HPPD variant protein, sufficient to maintain the viability of a cell in which it is expressed; and

(ii) catalytic activity that is more resistant to the herbicide than is wild type HPPD.

Also provided are nucleic acids encoding herbicide-resistant HPPD variants, DNA vectors comprising the nucleic acids, and cells comprising the variant HPPD-encoding vectors. Genes encoding herbicide-resistant HPPD variants can be used as

genetic markers, such as, for example, in plasmids and methods for the introduction and selection of any other desired gene.

In another aspect, the present invention provides a method for conferring herbicide resistance on a cell or cells, and particularly a plant cell or cells such as, for example, a seed. An HPPD gene, preferably the *Arabidopsis thaliana* HPPD gene, is mutated to alter the ability of an herbicide to inhibit the enzymatic activity of the HPPD. The mutant gene is cloned into a compatible expression vector, and the gene is transformed into an herbicide-sensitive cell under conditions in which it is expressed at sufficient levels to confer herbicide resistance on the cell.

Also contemplated are methods for weed control, wherein a crop containing an herbicide resistant HPPD gene according to the present invention is cultivated and treated with a weed-controlling effective amount of the herbicide.

Detailed Description of the Invention

The present invention encompasses isolated, purified, nucleic acids that encode plant 4-hydroxyphenylpyruvate dioxygenase (HPPD), expression systems in which enzymatically active HPPD is produced, and screening methods for identifying HPPD inhibitors.

The present invention also encompasses methods for screening for and producing plant HPPD variants that are resistant to the inhibitory action of herbicides, DNAs that encode these variants, vectors that include these DNAs, the HPPD variant proteins, and cells that express these variants. Additionally provided are methods for producing herbicide resistance in plants by expressing these variants and methods of weed control.

Isolation and Characterization of the Gene Encoding Arabidopsis HPPD

The present inventors have isolated and sequenced the gene encoding *Arabidopsis thaliana* HPPD, using the methods outlined below. Briefly, an *Arabidopsis thaliana* λ Yes cDNA library (Elledge et al., *Proc.Natl.Acad.Sci.USA* 88:1731, 1991) was screened using a PCR-based method (Amaravadi et al., *BioTechniques* 16:98, 1994).

Primers: A forward primer, designated ATHPPD1F (5'-CGTGCTCAGCGATGATCAGA-3') and a reverse primer, designated ATHPPD1R (5'-CGGCCTGTCACCTAGTGGTT-3') were synthesized based on an Arabidopsis EST

sequence (GenBank ID No: T20952) that showed homology to mammalian HPPD sequences.

The primers were evaluated in a polymerase chain reaction (PCR) using as template DNA a 1 μl aliquot (containing 3 x 10^6 pfu/ml) of the cDNA phage library. For PCR, a 50 μl reaction contained 1X PCR Buffer, 200 mM of each deoxynucleoside triphosphate, 1.25 units of AmpliTaq DNA Polymerase (all from Perkin Elmer), and 7.5 pmoles of each primer. The reaction mixture was heated to 95°C for 2 min and amplified using 35 cycles of: 95°C for 1 min, 48°C for 2 min, 72°C for 1 min 30 sec. This was followed by incubation at 72°C for 7 min. A fragment of the predicted size of 112 bp was produced. This fragment was cloned into the pCRII vector (TA Cloning Kit, Invitrogen) and sequenced, and was found to be identical to the *Arabidopsis* EST sequence (with the addition of 3 residues which had been undetermined in the reported sequence of the EST).

Library screening: The cDNA library was plated on 13 plates containing NZCYM agar at a density of 40,000 pfu/plate. The phage from each plate were eluted into SM, and aliquots from the 13 individual pools of phage were used as templates for PCR with the ATHPPD1F and ATHPPD1R primer pair. PCR conditions were as described above. (In the first round, 1 μl of each of the eluted phage pools was used as template, and 5 μl were used in subsequent rounds). In the first round, ten of the thirteen phage pools were positive by PCR. One of the positive pools was selected for further screening. In the second round, the eluates from 10 plates of 5,000 pfu/plate gave 1 positive pool. In the third round, 10 plates of about 20 pfu/plate gave 2 positive pools. The third round positive pools were plated out, and 36 individual plaques were picked and screened to find a single HPPD positive plaque. The insert-bearing plasmid was excised from this phage via the automatic subcloning properties of the vector. Restriction analysis indicated that this plasmid contained a 1.5 kb insert.

Sequence Analysis: Template DNA for sequencing was prepared using the Wizard DNA Purification System (Promega). Sequencing reactions were carried out using the fmol DNA Sequencing System (Promega), and sequence gels were run on Hydrolink Long Ranger (AT Biochem) gels. The insert of the HPPD-containing plasmid isolated from the cDNA library was sequenced using two primers that hybridize to the λYes vector on opposite sides of the XhoI cloning site in addition to a series of internal primers: ATHPPD1F ATHPPD1R as above; and

ATHPPD2F (5'-CTTCTACCGATTAACGAGCCAGTG-3');
ATHPPD2R (5'-CACTGGCTCGTTAATCGGTAGAAG-3');
ATHPPD3F (5'-TCCATCACATCGAGTTCTGGTGCG-3');
ATHPPD3R (5'-AAAAGGAATCGGAGGTCACCGGA-3');
ATHPPD4F (5'-CTGAGGTTAAACTATACGGCGA-3'); and
ATHPPD4R (5'-TCGCCGTATAGTTTAACCTCAG-3'). All sequence information was confirmed by sequencing both strands. Translation of the HPPD nucleotide sequence, sequence comparisons, and multiple sequence alignments were performed using the software in The Wisconsin Package, Version 8.0 (Genetics Computer Group, Madison, Wisconsin).

The results indicated that the 1.5 kb insert contains an open reading frame of 445 amino acids (Figure 1). A TFASTA search of the GenEMBL Database identified five known sequences as having partial homology: *Streptomyces* HPPD (U11864); rat F alloantigen (M18405), mouse HPPD (D29987); pig HPPD (D13390); and human HPPD (X72389). Direct pairwise comparisons of the *Arabidopsis* sequence with those mentioned above showed a 56% average similarity and a 37% average identity. Additionally, a number of conserved tyrosine and histidine residues, which have been proposed as metal-binding sites in mammalian HPPD (Ruetschi et al., *Eur.J.Biochem.* 205:459, 1992; Denoya et al., *J. Bacteriol.* 176:5312, 1994), are also observed in the *Arabidopsis* sequence.

Genomic organization of HPPD gene in *Arabidopsis*: Southern blot analysis was performed using genomic DNA prepared from *Arabidopsis* seedings according to the method of Dellaporta (Dellaporta et al., *Plant Mol.Biol.Rep.* 1:19, 1983). 10 μg of DNA were digested with the restriction enzymes BamHI, EcoRI and HindIII, after which the digests were separated on a 0.9% agarose gel, transferred to a Duralon-UV Membrane (Stratagene) using the VacuGene XI Vacuum blotting System (Pharmacia) and crosslinked using the Stratalinker UV Crosslinker (Stratagene). The HPPD probe was prepared by: (i) gel purifying (using GeneClean Kit, Bio 101, Inc.) the XhoI/SstI fragment from the digest of HPPD/λYes plasmid DNA. The fragment contains 50 bases of sequence upstream of the ATG start codon and extends to a position 55 bases upstream of the TGA stop codon; and (ii) labeling the fragment using the Prime-It Fluor Fluorescence Labeling kit (Stratagene). The labelled probe was hybridized to the membrane for 2 hours at 68°C using the QuikHyb Rapid Hybridization Solution (Stratagene). The membrane was

washed with 0.1X SSC/0.1% SDS once at room temperature and twice at 60°C, after which hybridization was visualized using the Illuminator Nonradioactive Detection System (Stratagene).

Only a single band hybridized to the probe under high stringency conditions in both the BamHI and HindIII digests. Two bands were observed in the EcoRI digest, reflecting the presence of an internal EcoRI site in the HPPD sequence. These results suggested that HPPD is encoded by a single-copy gene in *Arabidopsis*.

The entire HPPD coding sequence was then amplified from *Arabidopsis* genomic DNA using primers ATHPPD5F (5'-CCATGGGCCACCAAAACG-3') and ATHPPD5R (5'-CTGCAGTCATCCCACTAACTGTTTG-3'). The resulting genomic HPPD fragment, which was slightly larger than the corresponding cDNA fragment, was cloned into the pCRII vector (TA Cloning Kit, Invitrogen) and sequenced. A single intron of 107 bp was detected, located at nucleotide position 1163-1164 of the cDNA sequence.

Nucleic Acids, Vectors, Expression Systems, and Polypeptides

In practicing the present invention, many techniques in molecular biology, microbiology, recombinant DNA, and protein biochemistry such as these explained fully in, for example, Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis*, 1984, (M.L. Gait ed.); *Transcription and Translation*, 1984 (Hames and Higgins eds.); *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymology* (Academic Press, Inc.); and *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.), are used.

The present invention encompasses nucleic acid sequences encoding plant HPPD, enzymatically active fragments derived therefrom, and related HPPD-derived sequences from other plant species. As used herein, a nucleic acid that is "derived from" an HPPD sequence refers to a nucleic acid sequence that corresponds to a region of the sequence, sequences that are homologous or complementary to the sequence, and "sequence-conservative variants" and "function-conservative variants". Sequence-conservative variants are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. Function-conservative variants are those in which a given amino acid residue in HPPD has

been changed without altering the overall conformation and function of the HPPD polypeptide, including, but not limited to, replacement of an amino acid with one having similar physico-chemical properties (such as, for example, acidic, basic, hydrophobic, and the like). Fragments of HPPD that retain enzymatic activity can be identified according to the methods described herein, e.g., expression in *E. coli* followed by enzymatic assay of the cell extract.

HPPD sequences derived from plants other than *Arabidopsis thaliana* can be isolated by routine experimentation using the methods and compositions provided herein. For example, hybridization of a nucleic acid comprising all or part of the *Arabidopsis* HPPD sequence under conditions of intermediate stringency (such as, for example, an aqueous solution of 2X SSC at 65°C) to cDNA or genomic DNA derived from other plant species can be used to identify HPPD homologues. cDNA libraries derived from different plant species are commercially available (Clontech, Palo Alto, CA; Stratagene, La Jolla, CA). Alternatively, PCR-based methods can be used to amplify HPPD-related sequences from cDNA or genomic DNA derived from other plants. Expression of the identified sequence in, e.g., *E. coli*, using methods described in more detail below, is then performed to confirm that the enzymatic activity of the polypeptide encoded by the sequence corresponds to that of HPPD. Accordingly, HPPD sequences derived from dicotyledonous and monocotyledenous plants are within the scope of the invention.

The nucleic acids of the present invention include purine- and pyrimidine-containing polymers of any length, either polyribonucleotides or polydeoxyribonucleotides or mixed polyribo-polydeoxyribo nucleotides. This includes single- and double-stranded molecules, i.e., DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases. The nucleic acids may be isolated directly from cells. Alternatively, PCR can be used to produce the nucleic acids of the invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression.

The nucleic acids of the present invention may be flanked by natural *Arabidopsis* regulatory sequences, or may be associated with heterologous sequences,

including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.). Nucleic acids may contain one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. The nucleic acid may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the nucleic acid sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

The invention also provides nucleic acid vectors comprising the disclosed HPPD sequences or derivatives or fragments thereof. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clontech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), or pRSET or pREP (Invitrogen, San Diego, CA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g. antibiotic resistance, and one or more expression cassettes. Suitable host cells may be transformed/transfected/infected as appropriate by any suitable method including electroporation, $CaCl_2$ mediated DNA uptake, fungal infection, microinjection, microprojectile, or other established methods.

Appropriate host cells include bacteria, archebacteria, fungi, especially yeast, and plant and animal cells, especially mammalian cells. Of particular interest are *E. coli*, *B. Subtilis*, *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, *Schizosaccharomyces pombi*, *SF9* cells, C129 cells, 293 cells, *Neurospora*, and CHO

cells, COS cells, HeLa cells, and immortalized mammalian myeloid and lymphoid cell lines. Preferred replication systems include M13, ColE1, SV40, baculovirus, lambda, adenovirus, and the like. A large number of transcription initiation and termination regulatory regions have been isolated and shown to be effective in the transcription and translation of heterologous proteins in the various hosts. Examples of these regions, methods of isolation, manner of manipulation, etc. are known in the art. Under appropriate expression conditions, host cells can be used as a source of recombinantly produced HPPD-derived peptides and polypeptides.

Advantageously, vectors may also include a transcription regulatory element (i.e., a promoter) operably linked to the HPPD portion. The promoter may optionally contain operator portions and/or ribosome binding sites. Non-limiting examples of bacterial promoters compatible with *E. coli* include: *trc* promoter, β-lactamase (penicillinase) promoter; lactose promoter; tryptophan (trp) promoter; arabinose BAD operon promoter; lambda-derived Pl promoter and N gene ribosome binding site; and the hybrid tac promoter derived from sequences of the trp and lac UV5 promoters. Non-limiting examples of yeast promoters include 3-phosphoglycerate kinase promoter, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, galactokinase (GALI) promoter, galactoepimerase promoter, and alcohol dehydrogenase (ADH) promoter. Suitable promoters for mammalian cells include without limitation viral promoters such as that from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus (ADV), and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly A addition sequences, and enhancer sequences which increase expression may also be included. Sequences which cause amplification of the gene may also be desirable. Furthermore, sequences that facilitate secretion of the recombinant product from cells, including, but not limited to, bacteria, yeast, and animal cells, such as secretory signal sequences and/or prohormone pro region sequences, may also be included.

Nucleic acids encoding wild-type or variant HPPD polypeptides may also be introduced into cells by recombination events. For example, such a sequence can be introduced into a cell, and thereby effect homologous recombination at the site of an endogenous gene or a sequence with substantial identity to the gene. Other recombination-based methods, such as non-homologous recombinations or deletion of endogenous genes by homologous recombination, may also be used.

HPPD-derived polypeptides according to the present invention, including function-conservative variants of HPPD, may be isolated from wild-type or mutant *Arabidopsis* cells, or from heterologous organisms or cells (including, but not limited to, bacteria, fungi, insect, plant, and mammalian cells) into which an HPPD-derived protein-coding sequence has been introduced and expressed. Furthermore, the polypeptides may be part of recombinant fusion proteins. Alternatively, polypeptides may be chemically synthesized by commercially available automated procedures, including, without limitation, exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis.

"Purification" of an HPPD polypeptide refers to the isolation of the HPPD polypeptide in a form that allows its enzymatic activity to be measured without interference by other components of the cell in which the polypeptide is expressed. Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the HPPD protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against HPPD against peptides derived therefrom can be used as purification reagents. Other purification methods are possible.

The present invention also encompasses derivatives and homologues of HPPD polypeptides. For some purposes, nucleic acid sequences encoding the peptides may be altered by substitutions, additions, or deletions that provide for functionally equivalent molecules, i.e., function-conservative variants. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of similar properties, such as, for example, positively charged amino acids (arginine, lysine, and histidine); negatively charged amino acids (aspartate and glutamate); polar neutral amino acids; and non-polar amino acids.

The isolated polypeptides may be modified by, for example, phosphorylation, sulfation, acylation, or other protein modifications. They may also be

modified with a label capable of providing a detectable signal, either directly or indirectly, including, but not limited to, radioisotopes and fluorescent compounds.

Screening Methods to Identify HPPD Inhibitors/Herbicides

The methods and compositions of the present invention can be used to identify compounds that inhibit the function of HPPD and thus are useful as herbicides or as lead compounds for the development of useful herbicides. This is achieved by providing a cell that expresses HPPD and thereby produces homogentisic acid from 4-hydroxyphenylpyruvate (OHPP). Cell cultures expressing HPPD are incubated in the presence of test compounds to form test cultures, and in the absence of test compounds to form control cultures. Incubation is allowed to proceed for a sufficient time and under appropriate conditions to allow for interference with HPPD function. At a predetermined time after the start of incubation with a test compound, an assay is performed to monitor HPPD enzymatic activity. In a preferred embodiment, HPPD activity is monitored visually, by the appearance of red-brown pigments produced by oxidation and/or polymerization of homogentisic acid (La Du et al., in *Ochronosis. Pigments in Pathology*, M. Wolman (ed), Academic Press, NY, 1969). Alternatively, HPPD enzymatic activity may be monitored in cell extracts, using conventional assays such as that described in Example 1 below. Additional controls, with respect to both culture samples and assay samples, are also included, such as, for example, a host cell not expressing HPPD (e.g., a host cell transformed with an expression plasmid containing the HPPD gene in a reverse orientation or with no insert). HPPD inhibitory compounds are identified as those that reduce HPPD activity in the test cultures relative to the control cultures.

Host cells that may be used in practicing the present invention include without limitation bacterial, fungal, insect, mammalian, and plant cells. Preferably, bacterial cells are used. Most preferably, the bacterial cell is a variant (such as, e.g., the *imp* mutant of *E. coli*) that exhibits increased membrane permeability for test compounds relative to a wild-type host cell.

Preferably, the methods of the present invention are adapted to a high-throughput screen, allowing a multiplicity of compounds to be tested in a single assay. Such inhibitory compounds may be found in, for example, natural product libraries,

fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, and synthetic compound libraries. For example, synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle et al., *TibTech* 14:60, 1996). HPPD inhibitor assays according to the present invention are advantageous in accommodating many different types of solvents and thus allowing the testing of compounds from many sources.

Once a compound has been identified by the methods of the present invention as an HPPD inhibitor, *in vivo* and *in vitro* tests may be performed to further characterize the nature and mechanism of the HPPD inhibitory activity. For example, the effect of an identified compound on *in vitro* enzymatic activity of purified or partially purified HPPD may be determined as described in Example 1 below. Classical enzyme kinetic plots can be used to distinguish, e.g., competitive and non-competitive inhibitors.

Compounds identified as HPPD inhibitors using the methods of the present invention may be modified to enhance potency, efficacy, uptake, stability, and suitability for use in commercial herbicide applications, etc. These modifications are achieved and tested using methods well-known in the art.

Isolation of Herbicide-Resistant HPPD Variants

The present invention encompasses the isolation of HPPD variants that are resistant to the action of HPPD inhibitors/herbicides. The HPPD variants may be naturally occurring or may be obtained by random or site-directed mutagenesis.

In one embodiment, a population of cells or organisms expressing HPPD is mutagenized using procedures well-known in the art, after which the cells or organisms are subjected to a screening or selection procedure to identify those that are resistant to

the toxic effects of an HPPD inhibitor. The variant HPPD gene is then isolated from the resistant cell or organism using, e.g., PCR techniques.

In another embodiment, an isolated HPPD gene is subjected to random or site-directed mutagenesis *in vitro*, after which mutagenized versions of the gene are re-introduced into an appropriate cell such as, e.g., *E. coli*, and the cells are subjected to a selection or screening procedure as above.

The variant HPPD genes are expressed in an appropriate host cell, and the enzymatic properties of variant HPPD polypeptides are compared to the wild-type HPPD. Preferably, a given mutation results in an HPPD variant polypeptide that retains *in vitro* enzymatic activity towards 4-hydroxphenylpyruvic acid (OHPP), i.e., the conversion of OHPP to homogentisic acid (and thus is expected to be biologically active *in vivo*), while exhibiting catalytic activity that is relatively more resistant to the selected herbicide(s) than is wild-type HPPD. Preferably, when expressed in a cell that requires HPPD activity for viability, the variant HPPD exhibits (i) catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or catalytic activity in combination with any herbicide resistant HPPD variant protein also expressed in the cell, which may be the same as or different than the first HPPD variant protein, sufficient to maintain the viability of a cell in which it is expressed; and (ii) catalytic activity that is more resistant to the herbicide than is wild type HPPD.

Therefore, any one specific HPPD variant protein need not have the total catalytic activity necessary to maintain the viability of the cell, but must have some catalytic activity in an amount, alone or in combination with the catalytic activity of additional copies of the same HPPD variant and/or the catalytic activity of other HPPD variant protein(s), sufficient to maintain the viability of a cell that requires HPPD activity for viability. For example, catalytic activity may be increased to minimum acceptable levels by introducing multiple copies of a variant encoding gene into the cell or by introducing the gene which further includes a relatively strong promoter to enhance the production of the variant.

More resistant means that the catalytic activity of the variant is diminished by the herbicide(s), if at all, to a lesser degree than wild-type HPPD catalytic activity is diminished by the herbicide(s). Preferred more resistant variant HPPD retains sufficient catalytic to maintain the viability of a cell, plant, or organism wherein at the same

concentration of the same herbicide(s), wild-type HPPD would not retain sufficient catalytic activity to maintain the viability of the cell, plant, or organism.

Preferably the catalytic activity in the absence of herbicide(s) is at least about 5% and, most preferably, is more than about 20% of the catalytic activity of the wild-type HPPD in the absence of herbicide(s).

In the case of triketone-resistant variant HPPD, it is preferred that the HPPD variant protein has

(i) catalytic activity in the absence of said herbicide of more than about 20% of the catalytic activity of said wild-type HPPD; and

(ii) catalytic activity that is relatively more resistant to presence of triketone herbicides compared to wild type HPPD.

Herbicide-resistant HPPD variants can be used as genetic markers in any cell that is normally sensitive to the inhibitory effects of the herbicide on growth and/or pigment formation. In one embodiment, DNA encoding an herbicide-resistant HPPD variant is incorporated into a plasmid under the control of a suitable promoter. Any desired gene can then be incorporated into the plasmid, and the the final recombinant plasmid introduced into an herbicide-sensitive cell. Cells that have been transformed with the plasmid are then selected or screened by incubation in the presence of a concentration of herbicide sufficient to inhibit growth and/or pigment formation.

Chemical-resistant Plants and Plants Containing Variant HPPD Genes

The present invention encompasses transgenic cells, including, but not limited to seeds, organisms, and plants into which genes encoding herbicide-resistant HPPD variants have been introduced. Non-limiting examples of suitable recipient plants are listed in Table 1 below:

TABLE 1

RECIPIENT PLANTS

COMMON NAME	FAMILY	LATIN NAME
Maize	Gramineae	Zea mays
Maize, Dent	Gramineae	Zea mays dentiformis

COMMON NAME	FAMILY	LATIN NAME
Maize, Flint	Gramineae	Zea mays vulgaris
Maize, Pop	Gramineae	Zea mays microsperma
Maize, Soft	Gramineae	Zea mays amylacea
Maize, Sweet	Gramineae	Zea mays amyleasaccharata
Maize, Sweet	Gramineae	Zea mays saccharate
Maize, Waxy	Gramineae	Zea mays ceratina
Wheat, Dinkel	Pooideae	Triticum spelta
Wheat, Durum	Pooideae	Triticum durum
Wheat, English	Pooideae	Triticum turgidum
Wheat, Large Spelt	Pooideae	Triticum spelta
Wheat, Polish	Pooideae	Triticum polonium
Wheat, Poulard	Pooideae	Triticum turgidum
Wheat, Singlegrained	Pooideae	Triticum monococcum
Wheat, Small Spelt	Pooideae	Triticum monococcum
Wheat, Soft	Pooideae	Triticum aestivum
Rice	Gramineae	Oryza sativa
Rice, American Wild	Gramineae	Zizania aquatica
Rice, Australian	Gramineae	Oryza australiensis
Rice, Indian	Gramineae	Zizania aquatica
Rice, Red	Gramineae	Oryza glaberrima
Rice, Tuscarora	Gramineae	Zizania aquatica
Rice, West African	Gramineae	Oryza glaberrima
Barley	Pooideae	Hordeum vulgare
Barley, Abyssinian Intermediate, also Irregular	Pooideae	Hordeum irregulare
Barley, Ancestral Tworow	Pooideae	Hordeum spontaneum
Barley. Beardless	Pooideae	Hordeum trifurcatum
Barley, Egyptian	Pooideae	Hordeum trifurcatum
Barley, fourrowed	Pooideae	Hordeum vulgare polystichon
Barley, sixrowed	Pooideae	Hordeum vulgare hexastichon
Barley, Tworowed	Pooideae	Hordeum distichon
Cotton, Abroma	Dicotyledoneae	Abroma augusta
Cotton, American Upland	Malvaceae	Gossypium hirsutum
Cotton, Asiatic Tree, also Indian Tree	Malvaceae	Gossypium arboreum
Cotton, Brazilian, also, Kidney, and, Pernambuco	Malvaceae	Gossypium barbadense brasiliense

COMMON NAME	FAMILY	LATIN NAME
Cotton, Levant	Malvaceae	Gossypium herbaceum
Cotton, Long Silk, also Long Staple, Sea Island	Malvaceae	Gossypium barbadense
Cotton, Mexican, also Short Staple	Malvaceae	Gossypium hirsutum
Soybean, Soya	Leguminosae	Glycine max
Sugar beet	Chenopodiaceae	Beta vulgaris altissima
Sugar cane	Woody-plant	Arenga pinnata
Tomato	Solanaceae	Lycopersicon esculentum
Tomato, Cherry	Solanaceae	Lycopersicon esculentum cerasiforme
Tomato, Common	Solanaceae	Lycopersicon esculentum commune
Tomato, Currant	Solanaceae	Lycopersicon pimpinellifolium
Tomato, Husk	Solanaceae	Physalis ixocarpa
Tomato, Hyenas	Solanaceae	Solanum incanum
Tomato, Pear	Solanaceae	Lycopersicon esculentum pyriforme
Tomato, Tree	Solanaceae	Cyphomandra betacea
Potato	Solanaceae	Solanum tuberosum
Potato, Spanish, Sweet potato	Convolvulaceae	Ipomoea batatas
Rye, Common	Pooideae	Secale cereale
Rye, Mountain	Pooideae	Secale montanum
Pepper, Bell	Solanaceae	Capsicum annuum grossum
Pepper, Bird, also Cayenne, Guinea	Solanaceae	Capsicum annuum minimum
Pepper, Bonnet	Solanaceae	Capsicum sinense
Pepper, Bullnose, also Sweet	Solanaceae	Capsicum annuum grossum
Pepper, Cherry	Solanaceae	Capsicum annuum cerasiforme
Pepper, Cluster, also Red Cluster	Solanaceae	Capsicum annuum fasciculatum
Pepper, Cone	Solanaceae	Capsicum annuum conoides

COMMON NAME	FAMILY	LATIN NAME
Pepper, Goat, also Spur	Solanaceae	Capsicum frutescens
Pepper, Long	Solanaceae	Capsicum frutescens longum
Pepper, Oranamental Red, also Wrinkled	Solanaceae	Capsicum annuum abbreviatum
Pepper, Tabasco Red	Solanaceae	Capsicum annuum conoides
Lettuce, Garden	Compositae	Lactuca sativa
Lettuce, Asparagus, also Celery	Compositae	Lactuca sativa asparagina
Lettuce, Blue	Compositae	Lactuca perennis
Lettuce, Blue, also Chicory	Compositae	Lactuca pulchella
Lettuce, Cabbage, also Head	Compositae	Lactuca sativa capitata
Lettuce, Cos, also Longleaf, Romaine	Compositae	Lactuca sativa longifolia
Lettuce, Crinkle, also Curled, Cutting, Leaf	Compositae	Lactuca sativa crispa
Celery	Umbelliferae	Apium graveolens dulce
Celery, Blanching, also Garden	Umbelliferae	Apium graveolens dulce
Celery, Root, also Turniprooted	Umbelliferae	Apium graveolens rapaceum
Eggplant, Garden	Solanaceae	Solanum melongena
Sorghum	Sorghum	All crop species
Alfalfa	Leguminosae	Medicago sativum
Carrot	Umbelliferae	Daucus carota sativa
Bean, Climbing	Leguminosae	Phaseolus vulgaris vulgaris
Bean, Sprouts	Leguminosae	Phaseolus aureus
Bean, Brazilian Broad	Leguminosae	Canavalia ensiformis
Bean, Broad	Leguminosae	Vicia faba
Bean, Common, also French, White, Kidney	Leguminosae	Phaseolus vulgaris
Bean, Egyptian	Leguminosae	Dolichos lablab
Bean, Long, also Yardlong	Leguminosae	Vigna sesquipedalis
Bean, Winged	Leguminosae	Psophocarpus tetragonolobus

COMMON NAME	FAMILY	LATIN NAME
Oat, also Common, Side, Tree	Avena	Sativa
Oat, Black, also Bristle, Lopsided	Avena	Strigosa
Oat, Bristle	Avena	
Pea, also Garden, Green, Shelling	Leguminosae	Pisum, sativum sativum
Pea, Blackeyed	Leguminosae	Vigna sinensis
Pea, Edible Podded	Leguminosae	Pisum sativum axiphium
Pea, Grey	Leguminosae	Pisum sativum speciosum
Pea, Winged	Leguminosae	Tetragonolobus purpureus
Pea, Wrinkled	Leguminosae	Pisum sativum medullare
Sunflower	Compositae	Helianthus annuus
Squash, Autumn, Winter	Dicotyledoneae	Cucurbita maxima
Squash, Bush, also Summer	Dicotyledoneae	Cucurbita pepo melopepo
Squash, Turban	Dicotyledoneae	Cucurbita maxima turbaniformis
Cucumber	Dicotyledoneae	Cucumis sativus
Cucumber, African, also Bitter		Momordica charantia
Cucumber, Squirting, also Wild		Ecballium elaterium
Cucumber, Wild		Cucumis anguria
Poplar, California	Woody-Plant	Populus trichocarpa
Poplar, European Black		Populus nigra
Poplar, Gray		Populus canescens
Poplar, Lombardy		Populus italica
Poplar, Silverleaf, also White		Populus alba
Poplar, Western Balsam		Populus trichocarpa
Tobacco	Solanaceae	Nicotiana
Arabidopsis Thaliana	Cruciferae	Arabidopsis thaliana
Turfgrass	Lolium	
Turfgrass	Agrostis	

COMMON NAME	FAMILY	LATIN NAME
	Other families of turfgrass	
Clover	Leguminosae	

Expression of the variant HPPD polypeptides in transgenic plants confers a high level of resistance to herbicides including, but not limited to, triketone herbicides such as, for example, sulcotrione, allowing the use of these herbicides during cultivation of the transgenic plants.

Methods for the introduction of foreign genes into plants are known in the art. Non-limiting examples of such methods include *Agrobacterium* infection, particle bombardment, polyethylene glycol (PEG) treatment of protoplasts, electroporation of protoplasts, microinjection, macroinjection, tiller injection, pollen tube pathway, dry seed imbibition, laser perforation, and electrophoresis. These methods are described in, for example, B. Jenes et al., and S.W. Ritchie et al. In *Transgenic Plants, Vol. 1, Engineering and Utilization*, ed. S.-D. Kung, R. Wu, Academic Press, Inc., Harcourt Brace Jovanovich 1993; and L. Mannonen et al., *Critical Reviews in Biotechnology*, 14:287-310, 1994.

In a preferred embodiment, the DNA encoding a variant HPPD is cloned into a DNA vector containing an antibiotic resistance marker gene, and the recombinant HPPD DNA-containing plasmid is introduced into *Agrobacterium tumefaciens* containing a Ti plasmid. This "binary vector system" is described in, for example, U.S. Patent No. 4, 490,838, and in An et al., *Plant Mol.Biol.Manual* A3:1-19 (1988). The transformed *Agrobacterium* is then co-cultivated with leaf disks from the recipient plant to allow infection and transformation of plant cells. Transformed plant cells are then cultivated in regeneration medium, which promotes the formation of shoots, first in the presence of the

appropriate antibiotic to select for transformed cells, then in the presence of herbicide. In plant cells successfully transformed with DNA encoding herbicide-resistant HPPD, shoot formation occurs even in the presence of levels of herbicide that inhibit shoot formation from non-transformed cells. After confirming the presence of variant HPPD DNA using, for example, polymerase chain reaction (PCR) analysis, transformed plants are tested for their ability to withstand herbicide spraying and for their capabilities for seed germination and root initiation and proliferation in the presence of herbicide.

The methods and compositions of the present invention can be used for the production of herbicide-resistant HPPD variants, which can be incorporated into plants to confer selective herbicide resistance on the plants. Intermediate variants of HPPD (for example, variants that exhibit sub-optimal specific activity but high herbicide resistance, or the converse) are useful as templates for the design of second-generation HPPD variants that retain adequate specific activity and high resistance.

Herbicide resistant HPPD genes can be transformed into crop species in single or multiple copies to confer herbicide resistance. Genetic engineering of crop species with reduced sensitivity to herbicides can:

(1) Increase the spectrum and flexibility of application of specific effective and environmentally benign herbicides;

(2) Enhance the commercial value of these herbicides;

(3) Reduce weed pressure in crop fields by effective use of herbicides on herbicide resistant crop species and a corresponding increase in harvest yields;

(4) Increase sales of seed for herbicide-resistant plants;

(5) Increase resistance to crop damage from carry-over of herbicides applied in a previous planting;

(6) Decrease susceptibility to changes in herbicide characteristics due to adverse climate conditions; and

(7) Increase tolerance to unevenly or mis-applied herbicides.

For example, transgenic HPPD variant protein containing plants can be cultivated. The crop can be treated with a weed controlling effective amount of the herbicide to which the HPPD variant transgenic plant is resistant, resulting in weed control in the crop without detrimentally affecting the cultivated crop.

Description of the Preferred Embodiments

The following examples are intended to illustrate the present invention without limitation.

Example 1: Expression of *Arabidopsis* HPPD in *E. coli*

The following experiments were performed to demonstrate the production of high levels of enzymatically active *Arabidopsis* HPPD in *E. coli*.

A. ***Cloning and Bacterial Transformation:***

The HPPD coding sequence was cloned into the pKK233-2 expression vector (Clontech) so that the ATG initiation codon of HPPD was in-frame with the *trc* promoter using a PCR-based method. A primer designated ATHPPD6F (5'GAAATC*C*ATGGCACCAAAACG-3'), which hybridizes in the region of the HPPD start codon (in bold), includes a single base change (C from A, in italic) to generate an NcoI site (underlined). The primer ATHPPD6R (5'-CTTCTCCATGGTCATCCCACTAACTGT-3'), which hybridizes in the region of the

HPPD stop codon (in bold), includes an NcoI site outside the coding region (underlined).

A PCR reaction was performed using the above primers and, as template DNA, the HPPD sequence isolated from the cDNA library screen described above. The reaction mixture (100 μl) contained the following components: 2 ng plasmid DNA; 1X PCR buffer; 200 mM each deoxynucleotide triphosphate; 2.5 units AmpliTaq DNA Polymerase (Perkin Elmer); 13 pmol of primer ATHPPD6F; and 11 pmol of primer ATHPPD6F. The reaction mixture was heated to 95°C for 2 min, and then was amplified using 30 cycles of: 95°C, 1 min; 55°C, 2 min; 72°C, 1.5 min. This was followed by incubation at 72°C for 7 min.

A 1.3 kb PCR product was amplified. The fragment was resolved on a 1.2% Nu Sieve GTG gel (FMC) and was purified (GeneClean, Bio 101). The purified fragment was digested with NcoI and was ligated into NcoI-digested, alkaline phosphatase-treated pKK233-2 vector (Clontech). The ligation mixture was transformed into DH5α Library Efficiency Competent Cells (GibcoBRL). Transformants expressing HPPD were identified by the reddish-brown color produced when cultured overnight in LB with ampicillin.

Transformants were also prepared by transforming DH5α cells with empty pKK233-2 vector for use as a control in the enzyme assays.

B. *Production of Brown Pigment and Homogentisic Acid in E. coli*

Brown pigment formation was observed in colonies grown on solid media and in liquid cultures of *E. coli* transformed with the *Arabidopsis* HPPD gene. No similar brown pigmentation was associated with untransformed *E. coli* or with *E. coli* transformed with the control vector. Formation of the brown pigment (which exhibited a characteristic

absorption at 450 nm) was increased by supplementing the medium with tyrosine (Figure 2).

It is known that homogentisic acid turns brown when standing or when alkalinized and exposed to oxygen, due to the formation of an ochronotic pigment (La Du et al., in *Ocrhornosis. Pigments in Pathology*, M. Wolman (ed.), Academic Press, NY, 1969). Similar pigments are formed from the naturally-occurring secretion and oxidation of homogentisic acid in certain bacteria (Trias et al., *Can.J.Microbiol.* 35:1037, 1989; Goodwin et al., *Can.J.Microbiol.* 40:28, 1995). Thus, the occurrence of brown pigment suggested that *E. coli* cells transformed with the *Arabidopsis* HPPD gene as described above produce large amounts of homogentisic acid. Furthermore, since tyrosine is metabolized to hydroxyphenylpyruvate (thus providing additional substrate for HPPD), increased color development in the presence of increased tyrosine supports the conclusion that the brown pigment results from HPPD activity.

This was confirmed by measuring homogentisic acid directly using an HPLC-based method. The HPLC conditions for the determination of homogentisic acid were identical to those described by Denoya et al. (*J.Bacteriol.* 176:5312, 1994). The HPLC system consisted of a Waters 510 delivery module (Waters Assoc., Milford, MA), Waters 996 photodiode array detector, a WISP 710B automatic sampler, and a Waters 84O data integration system. A Phenomenex Spherisorb 5 ODS (l) CI8 reversed-phase column (5 mm particle size; 250 X 4.6 mm i.d.) was used, which was connected with a stainless steel guard column packed with C18 resin. The mobile phase (10 mM acetic acid:methanol; 85:15 v/v) was run at a flow rate of 1 ml/min. The wavelength was set at 292 nM. Culture broth samples (1 ml) were acidified by mixing with 100 ml of glacial acetic acid and were clarified by centrifugation. 50 ml of the mixture were injected on

the column. The peak corresponding to homogentisic acid was compared with a homogentisic acid standard for identification and quantitation.

The culture medium derived from overnight cultures of control *E. coli* cells showed no trace of homogentisic acid (Figure 3A). By contrast, HPPD-transformed *E. coli* produced a high level of homogentisic acid (Figure 3B). The peak eluting at 8 min co-migrated with authentic homogentisic acid and had an absorption spectrum identical with authentic homogentisic acid (insert).

C. *Assay of HPPD Activity*

E. coli transformants were treated with 0.1 mg/ml lysozyme in 50 mM potassium phosphate buffer (pH 7.3) at 30°C for 10 min. Cells were sonicated (3 times, 5 sec each, using a VibraCell sonicator, Sonics and Material, Inc., Danbury, CT) and the extract was subjected to centrifugation. The supernatant was desalted on an Econo-Pac 10DG column (Bio-Rad, Richmond, CA) that had been equilibrated with 5O mM phosphate buffer (pH 7.3). The desalted HPPD-containing extract was used for the HPPD assay.

HPPD enzymatic activity was determined by the capture of released $^{14}CO_2$ from ^{14}C-hydroxyphenylpyruvate (Schulz et al., *FEBS Letts.* <u>318</u>:162, 1993; Secor, *Plant Physiol.* <u>106</u>:1429, 1994). Reactions were performed in 20 ml scintillation vials, each capped with a serum stopper through which a polypropylene well containing 50 μl of benzethonium hydroxide was suspended. Each 450 μl reaction mixture contained: 50 mM potassium phosphate buffer (pH 7.3); 50 μl of a freshly prepared 1:1 (v/v) mixture of 150 mM reduced glutathione and 3 mM dichlorophenolindophenol; 2500 units of catalase; and bacterial extract (source of HPPD). Enzyme inhibitors were added where indicated. ^{14}C-

hydroxyphenylpyruvate (50 μl of a 2 mM solution), prepared according to the method of Secor (1994, *supra*), was added to initiate the reaction, which proceeded at 30°C for 30 min. The reaction was stopped by adding 100 μl 4 N sulfuric acid and the mixture was incubated for a further 30 min. The radioactivity trapped in benzethonium hydroxide was counted in a scintillation counter.

The results indicated that *E. coli* cells transformed with the *Arabidopsis* HPPD gene expressed very high levels of HPPD activity, i.e., 2.7 μmol/mg protein/hr. In contrast, HPPD activity was undetectable in untransformed or control *E. coli* cells. Furthermore, the HPPD activity was sensitive to inhibition by sulcotrione (Figure 4). Nearly complete inhibition of the activity was observed at more than 1 μM sulcotrione. The concentration of sulcotrione required to cause 50% inhibition of the activity was 100 nM.

<u>Example 2: High-throughput Screening of Test Compounds to Identify HPPD Inhibitors</u>

The following method is used in a high-throughput mode to identify HPPD inhibitors.

E. coli transformed with the *Arabidopsis* HPPD gene as described in Example 1 above is cultured overnight at 37°C in Luria Broth with 100 μg/ml ampicillin.

1 liter of molten LB agar containing 100 μg/ml ampicillin and 1 mM tyrosine is cooled to 50°C. 0.1 ml of the overnight *E. coli* culture is then added, and 150 ml of the mixture are poured into each 9 x 9 sterile Sumilon biotray (Vangard International, Neptune, NJ).

The plates are allowed to solidify and dry for 30 min. Test compounds (up to 25 μl) are applied to the test plate in sample wells (144 wells/plate, 5 cm diameter in

12 x 12 array) or in spots (6 x 96 compounds/plate). The plates are incubated overnight at 37°C.

The plates are scored by monitoring: (i) growth of *E. coli* and (ii) intensity of brown pigment. Zones in which the bacterial cells are viable but the pigment is reduced are scored as positive for HPPD inhibitors.

All patents, applications, articles, publications, and test methods mentioned above are hereby incorporated by reference.

Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed description. Such obvious variations are within the full intended scope of the appended claims.

Claims:

1. A purified isolated nucleic acid encoding plant HPPD.

2. A nucleic acid as defined in claim 1, derived from *Arabidopsis thaliana.*

3. A nucleic acid as defined in claim 2, wherein said nucleic acid is selected from the nucleic acid of SEQ ID NO:1, sequence-conservative variants thereof, and function-conservative variants thereof.

4. A DNA vector comprising the nucleic acid sequence of claim 3 operably linked to a transcription regulatory element.

5. A cell comprising a DNA vector as defined in claim 4, wherein said cell is selected from the group consisting of bacterial, fungal, plant, insect, and mammalian cells.

6. A cell as defined in claim 5, wherein said cell is a bacterial cell.

7. A cell as defined in claim 5, wherein said cell is a plant cell.

8. A seed comprising a cell as defined in claim 7.

9. An HPPD protein comprising a protein encoded by a DNA as defined in claim 2.

10. A method for identifying herbicides/HPPD inhibitors, said method comprising:

(a) providing a microbial cell expressing plant HPPD;

(b) incubating said cell in the presence of a test compound to form a test culture, and in the absence of a test compound to form a control culture;

(c) monitoring the level of homogentisic acid, or oxidation products thereof, in said test and control cultures; and

(d) identifying as a compound that inhibits HPPD any compound that reduces the level of homogentisic acid, or oxidation products thereof, in said test culture relative to said control culture.

11. A method as defined in claim 10, wherein said microbial cell is *E. coli*.

12. A method as defined in claim 10, wherein said monitoring comprises measuring the absorbance of said cultures at 450 nm.

13. A method as defined in claim 10, wherein said monitoring comprises detecting formation of a brown pigment.

14. A method for identifying herbicide-resistant HPPD variants, said method comprising:

(a) providing a population of cells expressing plant HPPD;

(b) mutagenizing said population of cells;

(c) contacting said mutagenized population of cells with an herbicide, under conditions inhibitory for the growth or pigment production of non-mutagenized cells;

(d) recovering cells resistant to the inhibitory effects of said herbicide on growth and/or pigment production; and

(e) sequencing HPPD-encoding nucleic acid from said recovered cells to identify herbicide-resistant HPPD variant.

15. A variant HPPD protein, wherein said protein is herbicide-resistant.

16. A variant HPPD protein as defined in claim 15, wherein said variant HPPD protein, when expressed in a cell that requires HPPD activity for viability, exhibits

(i) catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or catalytic activity in combination with any herbicide resistant HPPD variant protein also expressed in the cell, which may be the same as or different

than the first HPPD variant protein, sufficient to maintain the viability of a cell in which it is expressed; and

(ii) catalytic activity that is more resistant to the herbicide than is wild type HPPD.

17. A variant HPPD protein as defined in claim 15, wherein said protein is derived from *Arabidopsis thaliana*.

18. A nucleic acid encoding a variant HPPD protein as defined in claim 15.

19. A DNA vector comprising a nucleic acid as defined in claim 18.

20. A cell comprising a DNA vector as defined in claim 19, wherein said cell is selected from the group consisting of bacterial, fungal, plant, insect, and mammalian cells.

21. A cell as defined in claim 20, wherein said cell is a bacterial cell.

22. A cell as defined in claim 20, wherein said cell is a plant cell.

23. A seed comprising a cell as defined in claim 22.

24. A method for conferring herbicide resistance on a plant, said method comprising introducing into said plant a nucleic acid encoding an herbicide-resistant HPPD variant as defined in claim 16, under conditions in which said nucleic acid is expressed in said plant.

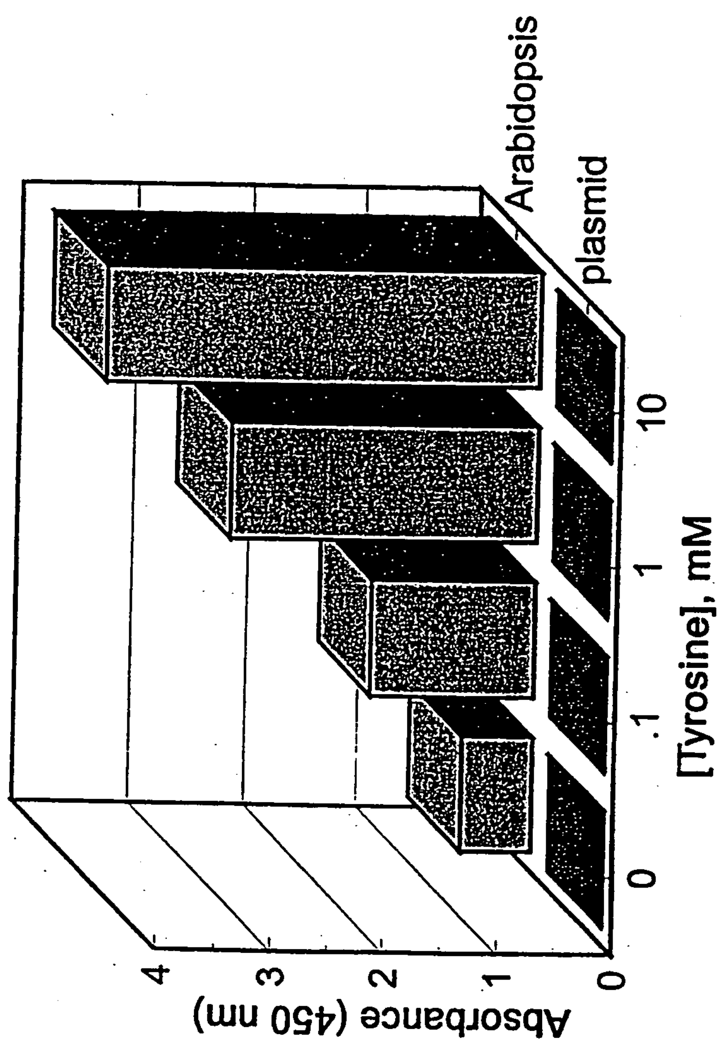
25. A method for weed control comprising cultivating a crop containing an herbicide-resistant HPPD gene in the presence of a week-controlling effective amount of said herbicide.

Figure 1

Sequence Alignment of Cloned HPPD Genes

```
AtHPPD   MGHQNAAVSENQNHDDGAASSPGFKLVGFSKFVRKNPKSDKFKVKRFHHIEFWCGDATNVARRFSWGLGMRFSAKSDLSTGNMVHASYLLTSGDLRFLFT
MOUSE    ..........................MTTYNNKGPKPERGRF..LHFHSVTFWVGNAKQAASFYCNKMGFEPLAYRGLETGSREVVSHVIKRGKIVFVLC
HUMAN    ..........................MTTYSDKGAKPERGRF..LHFHSVTFWVGNAKQAASFYCSKMGFEPLAYRGLETGSREVVSHVIKQGKIVFVLS
PIG      ..........................MTSYSDKGEKPERGRF..LHFHSVTFWVGNAKQAASYYCSKIGFEPLAYKGLETGSREVVSHVVKQDKIVFVFS
S.Aver   ........................MTQTTHHTPDTARQADPFPVKGMDAVVFAVGNAKQAA.HYSTAFGMQLVAYSGPENGSRETASYVLTNGSARFVLT

AtHPPD   APYSPSLSAGEIKPTTTASIPSFDHGSCRSFFSSHGLGVRAVAIEVEDAESAFSISVANGAIPSSPPIVLN..........EAVTIAEVKLYGDVVLRYV
MOUSE    SALN...PWNKEMG................DHLVKHGDGVKDIAFEVEDCDHIVQKARERGAKIVRE.......PWVEQDKFGKVKFAVLQTYGDTTHTLV
HUMAN    SALN...PWNKEMG................DHLVKHGDGVKDIAFEVEDCDYIVQKARERGAKIMRE.......PWVEQDKFGKVKFAVLQTYGDTTHTLV
PIG      SALN...PWNKEMG................DHLVKHGDGVKDIAFEVEDCDYIVQKARERGAIIVREEVCCAADVRGHHTPLDRAR....QVWEG...TLV
S.Aver   SVIKPATPWGHFLA...............DHVAEHGDGVVDLAIEVPDARAAHAYAIEHGARSVAE.......PYELKDEHGTVVLAAIATYGKTRHTLV

AtHPPD   SYKAEDTEKSEFLPGFERVEDASSFPLDYGIRRLDHAVGNVP..ELGPALTYVAGFTGFHQFAEFTADDVGTAESGLNSAVLASNDEMVLLPINEPVHGT
MOUSE    EKINYTGRFLPGFEAPTYKDTLLPKLPRCNLEIIDHIVGNQPDQEMQSASEWYLKNLQFHRFWSVDDTQVHTEYSSLRSIVVTNYEESIKMPINEPAPG.
HUMAN    EKMNYIGQFLPGYEAPAFMDPLLPKLPKCSLEMIDHIVGNQPDQEMVSASEWYLKNLQFHRFWSVDDTQVHTEYSSLRSIVVANYEESIKMPINEPAPG.
PIG      EKMTFCLDSRPQPSQTLLHRLLLSKLPKCGLEIIDHIVGNQPDQEMESASQWYMRNLQFHRFWSVDDTQIHTEYSALRSVVMANYEESIKMPINEPAPG.
S.Aver   DRTGYDGPYLPGYVAAA...PIVEPPAHRTFQAIDHCVGNVELGRMNEWVGFYNKVMGFTNMKEFVGDDIATEYSALMSKVVADGTLKVKFPINEPALA.

AtHPPD   KRKSQIQTYLEHNEGAGLQHLALMSEDIFRTLREMRKRSSIGGFDFMPSPPPTYYQNLKKRVGD..VLSDDQIKECEELGILVDRDDQGTLLQIFTKPLG
MOUSE    RKKSQIQEYVDYNGGAGVQHIALKTEDIITAIRHLRERGTE.....FLAAPSSYYKLLRENLKSAKIQVKESMDVLEELHILVDYDEKGYLLQIFTKPMQ
HUMAN    KKKSQIQEYVDYNGGAGVQHIALKTEDIITAIRHLRERGLE.....FLSVPSTYYKQLREKLKTAKIKVKENIDALEELKILVDYDEKGYLLQIFTKPVQ
PIG      KKKSQIQEYVDYNGGAGVQHIALKTEDIITAIRSLRERGVE.....FLAVPFTYYKQLQEKLKSAKIRVKESIDVLEELKILVDYDEKGYLLQIFTKPMQ
S.Aver   KKKSQIDEYLEFYGGAGVQHIALNTGDIVETVRTMRAAGVQ.....FLDTPDSYYDTLGEWVGDTRVPV....DTLRELKILADRDEDGYLLQIFTKPVQ

AtHPPD   DRPTIFIEIIQRVGCMMKDEEGKAYQSGGCGGFGKGNFSELFKSIEEYEKTLEAKQLVG*........
MOUSE    DRPTLFLEVIQRHNHQ...............GFGAGNFNSLFKAFEEEQALRGNLTDLEPNGVRSGM*
HUMAN    DRPTLFLEVIQRHNHQ...............GFGAGNFNSLFKAFEEEQNLRGNLTNMETNGVVPGM*
PIG      DRPTVFLEVIQRNNHQ...............GFGAGNFNSLFKAFEEEQELRGNLTDTDPNGVPFRL*
S.Aver   DRPTVFFEIIERHGSM...............GFGKGNFKALFEAIEREQEKRGNL*............
```

FIGURE 2


Arabidopsis
plasmid
Absorbance (450 nm)
0
1
2
3
4
0
.1
1
10
[Tyrosine], mM

FIGURE

Fig 3A.

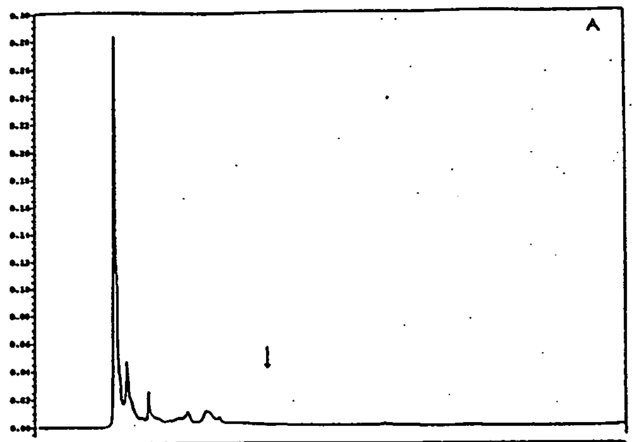


Fig 3B.

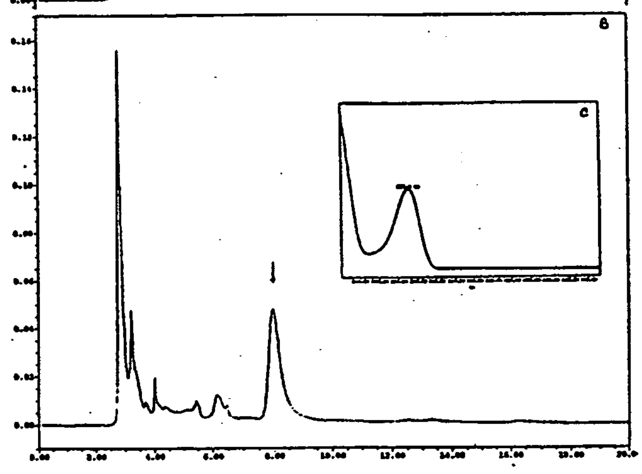
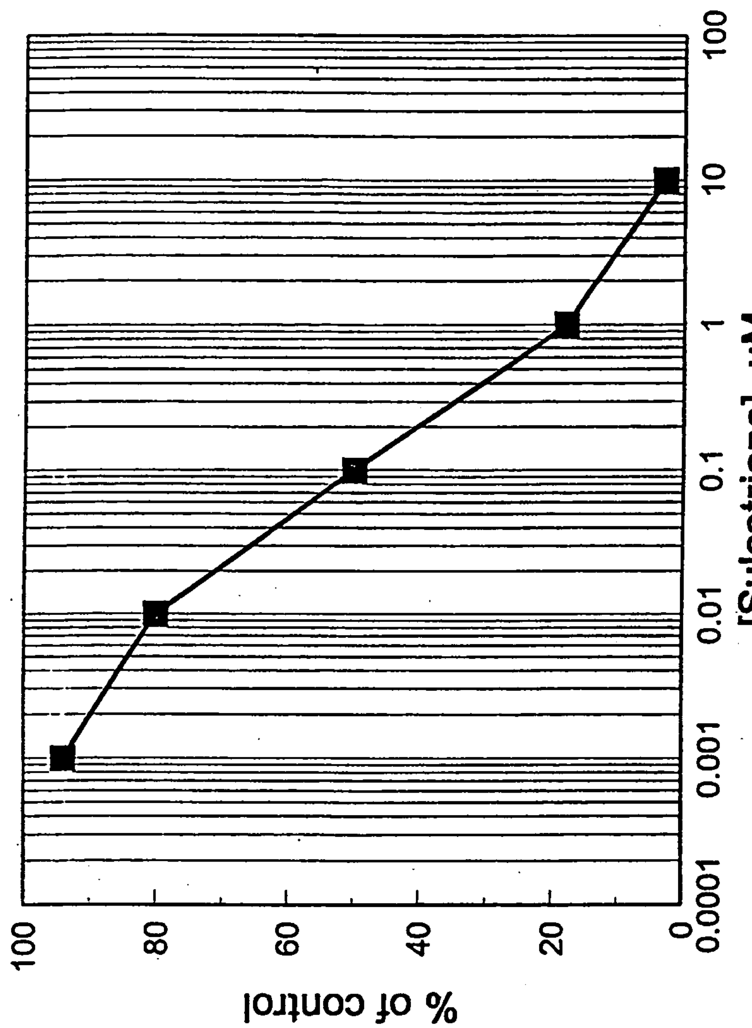


FiGuRE 4


% of control
100
80
60
40
20
0
0.0001
0.001
0.01
0.1
1
10
100
[Sulcotrione], uM

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14351

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 9/02, 1/20, 15/00, 15/09, 15/63, 15/82; C07H 21/04
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 189, 325, 410, 252.3, 252.33, 320.1; 536/23.1, 23.2, 23.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ---- Y,P	WO 96/38567 A2 (RHONE-POULENC AGROCHIMIE) 5 December 1996, abstract and claims.	1, 2, 14-25 ---- 10-13
Y	DE 43 05 696 A1 (HOECHST AG) 01 September 1994, see abstract and claims.	1, 10, 11, 14-16, and 18-25
Y,E	GARCIA et al. Subcellular localization and purification of a p-hydroxyphenylpyruvate dioxygenase from cultured carrot cells and characterization of the corresponding cDNA. Biochem. J. 01 August 1997, Vol. 325, pages 761-769, abstract	1, 10, 11, 1-16, and 18-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 NOVEMBER 1997	04 DEC 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	NASHAAT T. NASHED
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	BARTA et al. Purification and characterization of 4-hydroxyphenylpyruvate doxygenase from maize. Pestic. Sci. 02 October 1996, Vol. 48, pages 109-116, abstract.	1, 10, 14-16 and 18-25
X,P ---- Y,P	BARTLEY et al. 'Cloning and biochemical characterization of recombinant 4-hydroxyphenylpyruvate dioxygenase from Arabidopsis thaliana', Plant Physiol. Vol. 114, No. 3, Supp. [S], July 1997 (Rockville, MD), the abstract No. 1587, Session 62, Transgenics and Biotechnology.	1-9 ---- 10-25
A,E	LEE et al. The discovery and structural requirement of inhibitors of p-hydroxyphenylpyruvate dioxygenase. September-October 1997, Vol. 45, pages 601-609, entire document.	1-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14351

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 3-9
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claims are drawn to specific nucleic acid sequence of SEQ ID NO: 1. The description contains no nucleic acid sequences, and thus, the claims could not be searched.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14351

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/172.3, 189, 325, 410, 252.3, 252.33, 320.1; 536/23.1, 23.2, 23.6

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN: Medline, Caplus, Agricola, Scisearch, Lifesci, Biosis, Embase, Toxlit, Wpids
Search terms: 4-hydroxyphenylpyruvate dioxygenase, E.C. 1.13.11.27, E.C. 1.14.2.2, hydroxyphenylpyruvate hydroxylase, Plant

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1-9, drawn to purified nucleic acid sequence encoding plant 4-hydroxyphenylpyruvate dioxygenase (HPPD), vector containing DNA encoding HPPD, a cell containing a DNA encoding for HPPD and HPPD.

Group II, claims 10-13, drawn to a method for identifying herbicides which are inhibitors of HPPD.

Group III, claims 14-24, drawn to a method for identifying herbicide resistant HPPD (rHPPD), nucleic acid encoding rHPPD, vector containing DNA encoding rHPPD, a cell containing said DNA and a method of conferring herbicide resistance on plants.

Group IV, claim 25, drawn to a method of weed control.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical features of the inventions of Groups I-IV are (i) Group I: nucleic acid sequence encoding the wild-type HPPD, (ii) Group II, herbicides, (iii) Group III: herbicide resistant HPPD, and (iv) Group IV: weed control. Group I comprises the purified nucleic acid sequence encoding plant the wild-type HPPD, vector containing DNA encoding HPPD, a cell containing a DNA encoding HPPD and HPPD. Since the first claim of the Group defines the special technical feature, the nucleic acid sequence coding for the wild-type HPPD is the special technical feature for the invention which is different from those of Groups II-IV. Similarly, the special technical feature of Group II, which is different from those of Groups I, III and IV, are the herbicides because the claims are drawn to a method of identifying herbicides/inhibitors of HPPD. Group III encompasses a method for identifying rHPPD, nucleic acid encoding rHPPD, vector containing DNA encoding rHPPD, a cell containing said DNA and a method of conferring herbicide resistance on plants. Its special technical feature is rHPPD which is different from those of Groups I, II and IV. The special technical feature of weed control method of Group IV is the weed control which is different from those of Groups I-III. Thus, the claims are not so liked by a special technical feature within the meaning of PCT Rule 13.1 so as to form a single inventive concept.